CHAPTER II
MATERIALS AND METHODS

2.1 Materials

Chemicals and materials used in this study are described in Appendix.

2.2 Methods

2.2.1 Preparation methods

2.2.1.1 Versican isolation

Thirty five grams of mice brains were homogenized in warring blender using four volumes of the extraction buffer (0.5 M NaCl, 50 mM TrisHCl pH 7.5, 25 mM EDTA, 0.5% Nonidet P40, 1 mM PMSF, 1 µg/ml pepstatin, 1 µg/ml leupeptin) and stirred for 5 hr, at 4 ˚C. Then, the homogenized was centrifuged at 10,000 ×g for 1 hr and supernatant was collected. A solid Ammonium sulfate was added to supernatant on ice bath and stirred to precipitate protein out from supernatant. At initial step 30% Ammonium sulfate saturation, solution was centrifuged at 10,000 ×g at 4 ˚C for 30 min and supernatant was collected, then versican containing fractions were precipitated by increasing Ammonium sulfate concentration to 60%. The precipitate was allowed to form overnight at 4˚C. The 60% Ammonium sulfate containing supernatant was centrifuged at 10,000 ×g, at 4 ˚C for 40 min, after that precipitate was collected. The 60% Ammonium sulfate precipitate was reconstituted in a reconstituting buffer (50 mM Tri

sHCl pH 6.0, 10 mM EDTA, 6 M urea, 5% Nonidet P40, 1 mM PMSF, 1 µg ml pepstatin, 1 µg/ml leupeptin), then solid Cesium chloride (CsCl) was added to obtain 1.42 g/ml solution density. The solution was poured to polyallomer tube and applied to ultracentrifuge machine. The solution was centrifuged at 110,000 ×g, 10˚C, for 96 hr in swing rotor ultracentrifuge machine. The solution in centrifuge tube after centrifugation was fractionated into ten tubes, A1-A10 from bottom to top respectively. To identify these molecules, dot blot with anti CS-α chain monoclonal antibody was performed. The versican containing fractions were pooled and dialyzed extensively against distilled water.

2.2.1.2 WT and A subdomain less versican mouse embryonic fibroblasts culture preparation

The A subdomain less heterozygous male and female mice were mated, and let them gestated, these mice gestated WT, heterozygous A subdomain less, and homozygous A subdomain less versican type offsprings. After mating for 12 days, pregnant mice were euthanized by carbon dioxide gas. Embryos were isolated from pregnant mice, and soaked in Phosphate buffer saline (PBS) containing 1% penicillin/streptomycin. Placenta was removed, each embryo was separated, and placed in petri dish containing PBS with 1% penicillin/streptomycin. Embryo's tail was cut and collected in microcentrifuge tube for further genotyping, the rest was used to make fibroblasts culture. For genotyping, tail of each embryo was individually soaked in Protenase K overnight, afterwards genomic DNA was extracted by using DNAesy kit (Qiagen), and the extracted DNA was then analyzed by genomic PCR method.

PCR mixture for genomic PCR was prepared as described below.

	WT allele (µl)	Mutant allele (µl)
10× Extaq buffer	5	5
2.5 mM dNTP	5	5
Forward primer for WT or mutant	2.5	2.5
Reverse primer for WT or mutant	2.5	2.5
ddH_2O	29.5	29.5
ExTaq polymerase	0.5	0.5

Nine microliter of PCR mixture as described above was aliquoted to PCR tube , following with 1 µl DNA from embryo. The PCR reactions for WT and mutant alleles testing were performed by using different condition as described below.

	WT (˚C)	Mutant (˚C)
	94 (3 min)	94 (3 min)
35 cycles	94 (30 sec)	94 (30 sec)
	58 (30 sec)	62 (30 sec)
	72 (1 min)	72 (1 min 30 sec)
	72 (3 min)	72 (3 min)
	4 (∞ min)	4 (∞ min)

The PCR product was applied to 1% agarose gel, and electrophoresis was performed, then bands were visualized by Ethidium bromide, and embryo alleles were identified from those bands.

For fibroblasts culture preparation, PBS solution was removed from embryo soaking plate by aspiration carefully, embryo was roughly cut into small pieces by scissor. Trypsin/EDTA was added to those pieces, then incubated in incubator for 15-30 min. Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin was added to stop reaction. Medium was sucked out, and remaining tissues were crumbled up and passed though cell strainer to obtain single cell. Medium was then added and cells were cultured until reached to confluence. At the first and second passages, fibroblasts culture was collected and stored in liquid nitrogen for further assays.

2.2.2 Analytical methods

2.2.2.1 Cell proliferation assay

Cell proliferation rate was measured using a BrdU ELISA cell proliferation assay™ kit (Roche). Two thousand cells were plated in 96 well plates and cultured in DMEM containing 10% FBS, 1% penicillin/streptomycin for overnight in CO_2 incubator at 37 ˚C, 5% CO_2, 95% air, after that incubated with 10 µM BrdU labeling solution (100 µl) for 2 hr at 37 ˚C. DMEM containing 10% FBS, 1% penicillin/streptomycin was used as a negative control. The solution was removed, and then cells were fixed with 200 µl cell-fixing solution for 30 min at 25 ˚C and incubated with 100 µl of a peroxidase-conjugated anti-BrdU monoclonal antibody for 90 min at 25 ˚C. The solution was removed and washed for three times by 300 µl washing solution. 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution (100 µl/well) was added, and incubated at 25 ˚C, and then reaction was terminated by adding 25 µl 1 M HCl. The absorbance in each well was measured directly using a spectrophotometric microplate reader at a test wavelength of

450 nm and a reference wavelength of 650 nm. This provided us a measurement degree of cell proliferation.

2.2.2.2 Colony formation in soft agarose gel

Fibroblasts (1×10^4 cells) were suspended in 0.3% low-melting agarose in 3 ml DMEM containing 10% FBS, 1% penicillin/streptomycin and plated onto a solid underlaying of 2.0 ml 0.7% agarose in 35 mm culture dish and cultured. After 14 days, colonies were observed under a light microscope and photographed.

2.2.2.3 Tumor growth *in vivo*

Fibroblasts at the confluence were removed from tissue culture dish with trypsin/EDTA. Cells were washed twice with PBS, counted, and resuspended in PBS at 1×10^7 cell/ml cell density. Cells suspension (300 µl in PBS) was injected subcutaneously into BALB/c nu/nu slc mice (6 weeks old). After the injection, tumor formation was monitored every week.

2.2.2.4 β-galactosidase staining

Cells staining for β-galactosidase was performed following previously described by Dimri *et al.* (Dimri *et al.,* 1995) Fibroblasts were cultured in 35 mm culture dish until confluence, then washed in PBS and fixed in 2% formaldehyde for three min at room temperature. After washing, cells were incubated for 12 hr at 37˚C without CO_2 in freshly prepared staining buffer containing one mg/ml 5-bromo-4-chloro-3-indoyl β-D-galactoside (X-gal) in 0.1 M citric acid and 0.2 M Sodium phosphate solution, pH 6.0, 5 mM Potassium ferricyanide, 5 mM Potassium ferrocyanide, 150 mM NaCl, and 2 mM $MgCl_2$. Cells were then visualized and photographed under a light microscope.

2.2.2.5 Immunocytochemistry and immunohistochemistry

Immunocytochemistry for versican, fibronectin and laminin

Fibroblasts were cultured in DMEM containing 10% FBS, 1% penicillin/streptomycin in Lab-Tek II chamber slide until reached to confluence, subsequently medium was removed and rinsed with PBS. The culture slide was fixed in 10% buffered formalin for 30 min. The slide was washed in PBS three times, then slide was blocked with 10% goat serum for 1 hr, and it was immunostained with following specific antibodies: rabbit polyclonal anti-versican CS-α and CS-β (a mixture of ×1000 dilutions), rabbit polyclonal anti-laminin (×1000 dilutions), and rabbit polyclonal anti-fibronectin (×1000 dilutions). Slide was incubated overnight at 4 ˚C, then washed with PBS three times, an anti-rabbit IgG conjugated with biotin was added as the secondary antibody, slide was incubated at ambient temperature for 30 min, following with 3 washes with PBS. Streptavidin conjugated horseradish peroxidase (HRP-streptavidin) was added and incubated for 15 min at room temperature, before washing 3 times with PBS. Finally, Diaminobenzidine (DAB) solution was added to develop color, andstopped by deionized water. Culture slides were staind with hematoxylin for 10 sec, washed with deionized water and dipped in warm water for 5 min. Next, The slide was dipped in 70%, 90% and 100% ethanol for 3 min each, then anhydrous ethanol for 5 min, two times, and xylene for 3 min five times. The slide was mounted with mounting solution and enclosed with cover glass slip.

Immunocytochemistry for hyaluronan

Fibroblasts were cultured in DMEM containing 10% FBS in Lab-Tek II chamber slides until reached to confluence, subsequently medium was removed and rinsed with

PBS. The culture slide was fixed in 10% buffered formalin for 30 min. The slide was washed in PBS three times, and slide was blocked with 1% BSA for 1 hr, and immunostained with biotinylated-hyaluronan binding protein 2 μg/ml at 4 ˚C overnight. The slide was incubated overnight at 4 ˚C, following with 3 washed with PBS. HRP-streptavidin (×1000 dilutions) was added, slide was incubated at ambient temperature for 15 min. Afterwards, slide was washed with PBS three times and DAB solution was added to develop color. The reaction was stopped with deionized water and counterstained with hematoxylin for 10 sec, then washed with deionized water and dipped in warm water for 5 min. Next, The slide was dipped in 70%, 90% and 100% ethanol in deionized water for three min each, following with anhydrous ethanol for 5 min, 2 times, and xylene for 3 min, 5 times. The slide was mounted with mounting solution and enclosed with cover glass slip.

Immunocytochemistry for type I collagen

Fibroblasts were cultured in DMEM containing 10% FBS in Lab-Tek II chamber slide until reached to confluence. Subsequently, medium was removed and rinsed with PBS. The culture slide was fixed in 10% buffered formalin for 30 min, washed in PBS three times. Before Blocking, culture slide was treated with hyaluronidase 12.5 mg/ml in PBS for 20 min at ambient temperature. Slide was blocked with 10% goat serum for 1 hr, and immunostained with rabbit polyclonal anti-type I collagen (×1000 dilutions). Slide was incubated overnight at 4 ˚C, washed with PBS 3 times, then anti-rabbit IgG conjugated with biotin was added as the secondary antibody, slide was incubated at ambient temperature for 30 min. Afterwards, slide was washed with PBS 3 times, HRP-streptavidin was added and incubated for 15 min at room temperature. Slide was washed

with PBS 3 times, and DAB solution was added to develop color. The reaction was stopped with deionized water and counterstained with hematoxylin for 10 sec, washed with deionized water, dipped in warm water for 5 min. The slide was dipped in 70%, 90% and 100% ethanol for 3 min each, then anhydrous ethanol for 5 min 2, times, and xylene for 3 min 5 times. The slide was mounted with mounting solution and enclosed with cover glass slip.

Fluorescent immunocytochemistry for ERK1/2

Fibroblasts (2×10^5 cells) were grown in chamber slides for 24 hr, culture medium was changed from DMEM containing 10% FBS to 1% FBS and further grown for 18 hr. Afterwards, cells were fixed with 4% paraformaldehyde for 10 min at room temperature. After washing with PBS, cells were permeabilized with 0.2% Triton X-100 in PBS for 2 min at room temperature, blocked with 0.2% gelatin in PBS for 1 hr, andincubated with an anti-phosphoERK1/2 (×300 dilutions) at 4˚C overnight. After washing in PBS, cells were stained with Alexafluoro594-conjugated secondary antibody (×1000 dilutions) for ERK1/2 for 1 hr at room temperature, washed with PBS, mounted on glass slide, observed and photographed using a confocal laser induced fluorescent microscope (ZEISS).

Immunohistochemistry

Tumors from nude mice were fixed in 10% buffered formalin, and embedded in paraffin, then paraffin sections (3-5 µm) were made. The paraffin section slide was deparaffinized by dipping in xylene 5 min for 4 times, 100% ethanol 5 min 2 times, 90% ethanol 5 min, 80% ethanol 5 min, and 0.3 % H_2O_2 in absolute ethanol 5 min respectively. The slide was washed with distilled water, then PBS for 3 times. After that,

slide was stained with hematoxylin for 10 min, washed in running tap water for 20 min, counterstained with eosin for 2 min. Afterwards, the slide was dipped in 70%, 90% and 100% ethanol for 3 min each, then anhydrous ethanol for 5 min two times, and xylene for 3 min 5 times. The slide was mounted with mounting solution and enclosed with cover glass slip.

2.2.2.6 Sodium Dodesyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and immunoblot analysis

The SDS-PAGE was prepared as described. Glass plates for electrophoresis were assembled providing a 0.75 mm spacer. 30% acrylamide/0.8% bisacrylamide solution 7.5 ml, 7.5 ml 4x TrisHCl/SDS pH 8.8, and deionized water 15 ml were mixed together in 50 ml conical tube, added 10% ammonium persulfate 100 µl and N,N,N',N'-tetramethylethylenediamine (TEMED) 20 µl, swirled gently to mix, then separating gel mixture was obtained. The mixture was immediately poured into the assembled glasses. The mixture was overlaid with 100 µl water saturated with isobutyl alcohol, allowed the mixture to polymerize approximately 1 hr. Subsequently, stacking gel solution was prepared, 1.30 ml of 30% acrylamide/0.8% bisacrylamide, 2.50 ml of 4x TrisHCl/SDS, pH 6.8, and 6.10 ml deionized water were mixed together in 50 ml conical tube, 50 µl of 10% ammonium persulfate and 10 µl TEMED were added and swirled gently to mix. Water saturated with isobutyl alcohol was removed and the stacking gel mixture was filled upto the top instead. Comb was carefully placed to stacking gel mixture immediately, allowed the mixture to polymerized approximately 1 hr. Comb was removed and rinsed with running buffer containing 0.025 M Tris, 0.19 M glycine and 0.1% SDS. Gel was assembled with electrophoresis chamber, the upper and lower

electrophoretic chambers were filled with running buffer. Samples were prepared as described. Cells were lysed in 50 mM Tris-HCl, 2 mM EDTA, 2 mM EGTA, 150 mM NaCl, 2% Triton X-100 and protease inhibitors (Protease inhibitor cocktail tablets, Roche, USA). The cell lysates and media were centrifuged at 13,000 ×g for 10 min at 4 ˚C, supernatants were collected. Protein concentration was determined using the bicinchoninic acid (BCA) assay kit (Pierce, France), samples at an equal protein amount were mixed with equal volume of sample buffer containing 2x concentrated sample buffer consisting of 2% SDS, 20% glycerol, 20 mM Tris-HCl, pH 6.8, 2 mM EDTA, 2% 2-mercaptoethanol, 0.1 mg/ml of bromophenol blue to serve as a tracking dye. The mixture was boiled for 5 min in order to denature protein. The samples were subjected to gel, andelectrophoresis was performed under a constant current at 15 mA at room temperature. The proteins in gel were electro-transferred to a polyvinylidene difluoride (PVDF) membrane (membrane was rinsed with methanol, deionized water and soaked in transfer buffer containing 0.025 M Tris-base, 0.192 M glycine, and 20% methanol before use). Blotting was performed using constant voltage at 6 V at 4 ˚C overnight, subsequently the membrane was soaked in 5% skimmed milk in PBS containing 0.1% Tween 20 for 1 hr at room temperature for blocking. The membrane was treated with primary antibodies at 4 ˚C overnight. After washing three times with PBS containing 0.1% Tween 20, the membrane was treated with corresponding secondary antibodies at room temperature for 1 hr. After washing three times with PBS containing 0.1% Tween 20, the signal was detected with ECL reagents (Amersham Biosciences, USA). When necessary, the blots were stripped in stripping buffer (Pierce, France), and the membrane was used for immunoblot analysis using another antibody.

2.2.2.7 Chondroitin sulfate chain analysis

Cells were cultured in 60 mm culture dishes up to the confluence, then media and cells were collected. Cell lysate collected by a Cytobuster reagent (Novagens) was subjected to centrifugation at 13,000 ×g 4 ˚C for 10 min and supernatant was collected. The supernatant or cultured medium was applied to a 0.3 ml DEAE-Sephacel column, equilibrated with the equilibration buffer (50 mM Tris-HCl, pH 7.2, 0.1 M NaCl). After washing with 3 ml equilibration buffer, proteoglycan fraction was eluted with elution buffer (50 mM Tris-HCl, pH 7.2, 2 M NaCl). Three volumes of 95% ethanol containing 1.3% potassium acetate were added to the eluate, and the solution was chilled at -20 ˚C overnight, and centrifuged at 13,000 ×g for 30 min at 4 ˚C. The pellet was washed with 98% ethanol twice, and dried. The pellet was reconstituted in deionized water and protein concentration in the proteoglycan precipitate pellet was determined by BCA assay. The equal amount of proteoglycan was dissolved in chondroitinase ABC buffer (20 mM Tris-HCl pH 8.0, 20 mM sodium acetate, 0.02% BSA), digested with chondroitinase ABC (5 mU/ml) at 37 ˚C for 3 hr, reaction was stopped by boiling at 100 ˚C for 5 min. The sample was pretreated by added 100 µl distilled water (HPLC grade) to centrifugal filter and centrifuged at 14,000 rpm for 4 min and removed flow through twice, then added sample and centrifuged at 14,000 rpm for 15 min and collected flow through for further analysis by high performance liquid chromatography (HPLC). The sample was added to HPLC vial tube and performed HPLC, conditioned to operate HPLC was mentioned below.

Buffer for HPLC

Buffer A: distilled water (HPLC grade)

Buffer B: 0.2 M NaCl

Buffer C: 10 mM tetra-N-butylammonium hydrogensulfate

Buffer D: acetonitrile 50%

Post column reaction reagent

NaOH 0.25 M

cyanoacetemide 0.5% in distilled water (HPLC grade)

HPLC system

Helium gas for driven buffer set at 2 kg/cm^2

Flow rate set at 1.1 ml/min

Composition

Buffer A = 70 %, Buffer B = 1 %, Buffer C = 12 %, Buffer C = 17 %

Sparge

Buffer A = 30 %, Buffer B = 30 %, Buffer C = 30 %, Buffer D = 30 %

System pressure 800 psi

Unsaturated CS disaccharide products were analyzed by fluorometric post-column type HPLC, and its content was calculated from standard curve of standard chondroitin sulfate derived disaccharides.

2.2.2.8 Real-time reverse transcription-polymerase chain reaction (real-time RT-PCR)

PolyA RNA was prepared from cultured fibroblasts using MicroFast Track™ kit (Invitrogen, USA), and cDNA was made using reverse-transcription reaction as described below by using a SuperScript III First-Strand synthesis System kit (Invitrogen, USA). The RNA sample 8 µl was mixed to 50 µg/ml random hexamer 3 µl, 10 mM dNTP 1 µl,

deionized water 1 µl. The mixture was incubated at 65 ˚C for 5 min, then placed on ice for 1 min. Two µl of the mixture was added to 9 µl reaction mixture containing 10× reverse transcriptase, 25 mM $MgCl_2$ 4 µl, 0.1 M Dithiothreitol 2 µl, RNase inhibitor 1 µl, and mixed gently. After that, the mixture was incubated at 25 ˚C for 2 min,1 µl reverse transcriptase was added and PCR reaction was performed as follow

65 ˚C	10 min	3 cycles
42 ˚C	120 min	
70 ˚C	15 min	

Reaction mixture was placed on ice and 1 µl RNase H was added and incubated at 37 ˚C 20 min to remove RNA residues from cDNA. The cDNA was used as a template for real time PCR by using the Taqman Prism 7700 machine (Applied Biosystems). The sequences of the probe and a set of primers for mouse versican were: forward primer, 5'-CCAGTGTGAACTTGATTTTGATGAA-3'; reverse primer, 5'-AACATAACTTGGGAGACAGAGACATCT-3'; Taqman probe, 5'-CACTCTAACCCTTGTCGGAATGGT-3'. The reaction mixtures for real time PCR were shown below.

	Versican (µl)	GAPDH (µl)
cDNA	2.0	2.0
10 pM Taqman probe	0.2	0.8
10 pM forward primer	0.2	1.0
10 pM reverse primer	0.2	1.0
Deionized water	7.4	5.2

2x master mix solution	10.0	10.0

2.2.2.9 Sandwich ELISA for hyaluronan content determination

For the HA assay, Cells portion at confluence and the conditioned medium were collected, cells portion was placed in 0.5 ml of 0.15 M Tris-HCl pH 7.3, 0.15 M NaCl, 10 mM $CaCl_2$ and 5 mM deferoxamine mesylate containing 10 units of protease K and incubated for 2 hr at 55 ˚C in order to prevent HA from hyaluronidase digestion. The samples were centrifuged at 14,000 ×g for 25 min at 4 ˚C, the supernatants were collected for analysis. HA concentration was measured using sandwich enzyme-linked immunosorbent assay (ELISA). Microtiter plates were coated with 50 µl of 0.25 µg/ml HABP at 4 ˚C overnight, and then were blocked with 2% BSA in PBS-Tween for 1 hr at 37 ˚C. To each well, the samples or various concentrations of HA (HA standards) were added and incubated for 1 hr 37 ˚C, then a biotinylated HABP solution was added and incubated for another 1 hr at 37 ˚C. After incubation, the plates were washed and further incubated with peroxidase-conjugated streptavidin. Finally, a color was developed, the reaction was stopped with 1 M HCl, and the absorbance was measured at 450 nm by using spectrophotometric microplate reader.

2.2.2.10 Cell adhesion assay

Ninety-six well plates were coated with various kinds of extracellular matrix molecules including laminin, fibronectin or type I collagen 5 µg/ml 50 µl in PBS, and PBS solution was used as a negative control. Plates were incubated at 4˚C overnight. Residual protein-binding sites were blocked by incubating the plates in 1.2% BSA for 2 hr at 37 ˚C. Cells were suspended in DMEM/F12 (1×10^5 cells/ml), cells suspension 100 µl was plated and incubated in CO_2 incubator for 1 hr. After the incubation, the

unattached cells were removed by gently shaking for 5 sec and washed twice with PBS. The attached cells were incubated in 110 µl DMEM/F12 containing 10% Alamar blue dye for 3 hr at 37˚C. The number of the attaching cells was estimated by measuring an absorbance at 570/600 nm. The number of cells was calculated using standard plates containing known number of cells with various numbers of cells (2×10^4 cells and diluted two-folds for 4 times), then performed under the same condition of the tested plates but cells were not removed from plates.

2.2.2.11 Attachment assay

Fibroblasts were cultured in tissue culture plate until reached to confluence, cells were washed with PBS twice, trypsin/EDTA was added and incubated in CO_2 incubator for 3 min. Cells were harvested and washed with PBS twice, then cell number was counted and suspended in DMEM containing 10% FBS, 1% Penicillin/Streptomycin. Cells at 1×10^5 cells were plated to 35 mm culture dish and incubated in CO_2 incubator, photographed at 30 min, 1, 2, 4, and 7 hr after incubation.

2.2.2.12 Protein assay

To determine protein concentration in sample, bicinchoninic acid (BCA) assay (Pierce) was performed. Firstly, standard bovine serum albumin (BSA) was prepared to make calibration curve, two mg/ml BSA was diluted to achieve concentrations ranged from 0.5 -200 µg/ml, then reagent A (sodium carbonate, sodium bicarbonate, and sodium tartrate in 0.2 M sodium hydroxide), reagent B (4% bicinchoninic acid) and reagent C (4% cupric sulfate pentahydrate) were mixed thoroughly at ratio 50:48:2 respectively to make working reagent. Each 25 µl of standard and unknown sample was placed into microplate well, BCA working reagent 200 µl was added and plate was mixed thoroughly

on plate shaker for 30 sec. Plate was covered and incubated at 37 ˚C for 30 min, then cooled down to room temperature and measured at absorbance 562 nm in microplate reader. Concentration of sample was calculated from calibration curve of standard BSA.

2.2.2.13 Collagen assay

Collagen content in conditioned medium and cell layer was determined by the following method. Sircol collagen assay (Biocolor) was a method to determine collagen content. Conditioned medium of fibroblasts at confluence was collected and cells layer remaining on cultured dish was washed with PBS and collected using cell scrapper, 2 ml PBS solution was added into collected cells layer and mixed thoroughly. This assay did not use trypsin/EDTA in order to prevent collagen destruction. The collected medium and cell layer were centrifuged at 14,000 rpm for 15 min, supernatant was collected. Standard solutions of collagen were placed into microcentrifuge tube at 12.5, 25, and 50 µg content, deionized water was added upto 100 µl in each tube. Sample 100 µl was placed into microcentrifuge tube. One ml of Sircol dye reagent (Sirius Red in cupric acid) was added to standard collagen and sample solution, mixed gently at room temperature for 30 $\pm$ 5 min. The mixture was centrifuged at 10,000 rpm for 10 min to obtained collagen-dye pellet. Supernatant was removed and any other dye solutions attaching to microcentrifuge tube wall were removed by cotton bud. Alkaline reagent (0.5 M NaOH) was added to release dye from collagen, solution was mixed thoroughly and left for 5 min, 200 µl solution was transferred to each well in 96 wells plate and measured absorbance at 540 nm within 3 hr. Collagen content can be measured from calibration curve derived from standards collagen.

2.2.2.14 Treatment with an anti-CD44 antibody and hyaluronidase

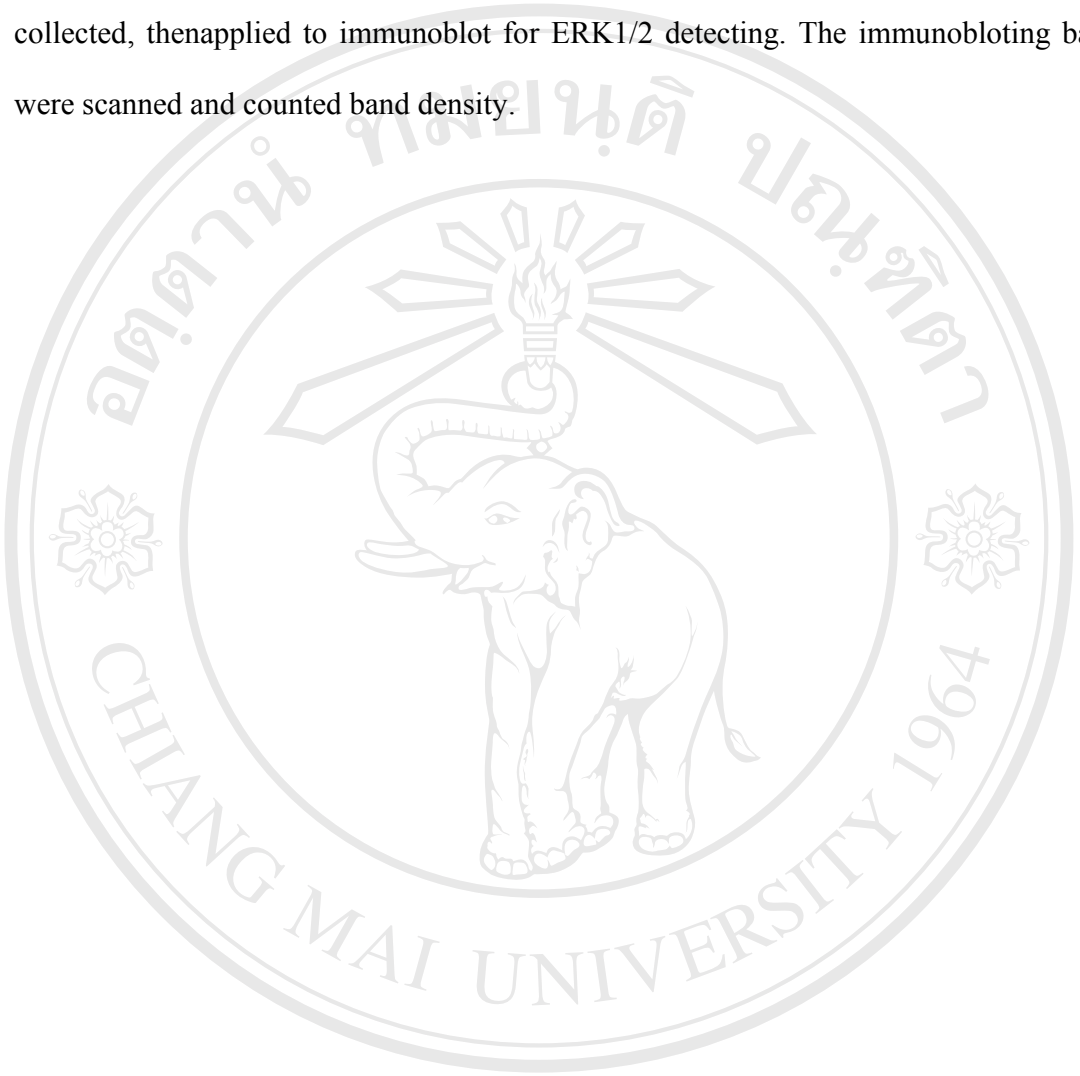
Fibroblasts (2×10^5 cells) were grown in chamber slides for 24 hr, andcultured medium was changed to 1% FBS and further grown for 18 hr. The anti-CD44 monoclonal antibody (Cedar Lane Laboratories, Canada) was added to the fibroblasts culture at final concentrations of 1.0 – 5.0 μg/ml. The cells were cultured for 1 hr, and fixed with 4% paraformaldehyde for 10 min at room temperature. After washing with PBS, cells were permeabilized with 0.2% TritonX-100 in PBS for 2 min at room temperature, blocked with 0.2% gelatin in PBS for 1 h, and then incubated with an anti-phosphoERK1/2 (×300 dilutions, Cell signaling, USA) at 4˚C overnight. After washing in PBS, cells were stained with Alexafluoro594-conjugated secondary antibody (×1000 dilutions, Molecular Probes) for 1 hr at room temperature, washed with PBS, mounted on microscope slide, and observed using a confocal laser induced microscope (ZEISS).

For hyaluronidase treatment, the WT fibroblasts at the confluence in 6-well plates were used. The medium was replaced with fresh medium containing 1% FBS and the cells were cultured for 18 hr. Then bovine testicular hyaluronidase (Sigma, USA) at final concentrations of 0.2 and 2.0 mg/ml was added, and the cells were cultured for 30 min and 2 hr. Cell lysates were collected and immunoblot was performed to detect phosphoERK1/2, ERK1/2, and actin. In other experiments, the cultures were treated with 0.2 and 2.0 mg/ml hyaluronidase alone, or together with 5.0 μg/ml anti-CD44 antibody for 2 hr, following with immunoblot as described above.

2.2.2.15 Treatment with a hyaluronan fragments.

The WT fibroblasts were cultured in 6 well plates until reached to confluence, the medium was replaced with fresh medium without serum, then cultured for 2 hr. HA with

molecular sizes of 1,000 kDa, 104 kDa, 8.2 kDa, at final concentrations of 5, 50, 150 μg/ml was added to the culture, and cultured for further 16 h, and the cell lysates were collected, thenapplied to immunoblot for ERK1/2 detecting. The immunobloting bands were scanned and counted band density.



ml pepstatin, 1 μg/ml leupeptin), then solid Cesium chloride (CsCl) was added to obtain 1.42 g/ml solution density. The solution was poured to polyallomer tube and applied to ultracentrifuge machine. The solution was centrifuged at 110,000 ×g, 10˚C, for 96 hr in swing rotor ultracentrifuge machine. The solution in centrifuge tube after centrifugation was fractionated into ten tubes, A1-A10 from bottom to top respectively. To identify these molecules, dot blot with anti CS-α chain monoclonal antibody was performed. The versican containing fractions were pooled and dialyzed extensively against distilled water.